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FULL PAPER

Analysis method of phytosterol constituents from vegetable to facilitate product development

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Abstract

In 2003, The United States Food and Drug Administration (USFDA) has approved food that contains at least 400 mg plant sterol eaten twice with meals for a daily total intake of at least 0.8 g, as part of a diet low in saturated fat and cholesterol. This recommendation is part of an effort to reduce the risk of heart disease. In this study, four types of phytosterol constituents which include stigmasterol, β-sitosterol, campesterol and stigmastanol have been used as standard reference since all the secondary metabolite constituents were reported widely found in vegetables. For analysis purposes, all the constituents were extracted either using solid-liquid extraction or liquid-liquid extraction. In the case of a solid sample, solid-liquid extraction with chloroform as a solvent is more suitable, while for a liquid sample, liquid-liquid extraction is more favourable. The detection of the phytosterol constituents was conducted via a gas chromatography-mass spectrometer (GCMS). The developed method will be useful for screening and selection of potential vegetables with phytosterol for food product development. In addition, the method could be used for monitoring batch to batch production of products in order to ensure quality.

1. Introduction

Vegetables are one of the important food agricultural productions in Malaysia besides cocoa, paddy and fruits. Until 2020, almost 65,000 hectares of Malaysian land which include Peninsular, Sabah and Sarawak were covered with vegetables. From statistical information, Peninsular Malaysia has the biggest arable land for vegetables accounted for more than 53,000 hectares followed by Sarawak and Sabah (Department of Agriculture Malaysia, 2020). To date, there are approximately twenty-seven major vegetables were recorded domestically planted nationwide.

Vegetables contain abundant vitamins, minerals and other important secondary metabolites that can promote better health in our daily activities. As proven scientifically, primary metabolites from vegetables are very important for our healthy growth, and vegetables also contain some secondary metabolites, especially phytosterol constituents. These constituents have been reported to play an important role in lowering cholesterol levels (Trautwein *et al*., 2018). As mentioned in the literature, four major phytosterol constituents are known as campesterol, stigmasterol, β-sitosterol and stigmastanol have been identified from the vegetables (Bacchetti *et al*., 2011; Yang *et al*., 2019). Some vegetables are consumed fresh such as salads and juices, and some vegetables have a very short shelf-life and freshness, and most vegetable crop materials are utilized as processed food products.

A high-tech phyto-food production involving very high-quality raw materials and its clean and proper production system is needed, thus a proper quality control protocol should be included in the analysis work and finally to be established as a methodology of phytochemistry analysis. In this study, a new method for phytochemical analysis of phytosterol constituents has been developed in order to establish product development containing those phytosterols. This quality procedure is important in order to ensure every batch of products is fulfilled with value-added in the manufacturing of the vegetable industry.

2. Materials and methods

2.1 General

Four analytical standards of phytosterols which

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include stigmasterol, β-sitosterol, campesterol and stigmastanol were purchased from Sigma Aldrich, USA. The analytical grade solvents purchased were chloroform and methanol (Merck) were used for extraction and standard preparation. The phytosterols analysis was carried out using a Gas Chromatography-Mass Spectrometer (GCMS) Thermo Scientific consisting of an Electron Impact (EI) source system.

2.2 Preparation and extraction of standard/sample

Each phytosterol standard and solid form sample were weighed in a quantity of 1.0 mg and 100 g, respectively. Chloroform was used for extraction due to its solvent solubility as reported by Araújo *et al*. (2013). The solid sample was dried at 40°C for 48 hrs in order to remove moisture. The sample (spinach) was grounded and macerated with chloroform for 24 hrs. Subsequently, the macerate was filtered using a filter paper Whatman No. 1 and the solvent was evaporated using a rotavapor at 45°C. For a liquid sample, the phytosterol constituents were extracted using a liquid-liquid extraction technique. A 100 mL of sample was added with methanol and chloroform with a ratio of 1:1:1. The mixture was transferred into a separating funnel, then continuously shaken and allowed to stand for 10 mins. Subsequently, the lower layer of the mixture was collected and evaporated using a rotavapor at 45°C. All the phytosterol standards were dissolved in 1.0 mL chloroform. Then, the prepared solutions were diluted five times using a serial dilution from 500 ppm to 30 ppm and each were labelled as a working solution. The extract and diluted phytosterol standard solutions were stored at 4°C prior to GC-mass analysis.

2.3 Detection and quantification

The GCMS analysis method was conducted based on the method developed by Munoz and Ramos (2016) for analysis of selected phytosterol. The instrument was equipped with a TG-5MS capillary column (30 m length × 0.25 mm I.D, 0.25 µm diameter). For each analysis, 2 µL of the sample was injected into the GCMS system. The acquisition parameters for the EI ion source were set at a minimum MS 50 and a maximum at MS 600. A gas temperature was set at 250°C, carrier flow rate at 0.8 mL/min. The initial column temperature was set at 150°C for 5 mins, then it was increased by 50°C/min until 320°C and the final temperature was held for 10 mins. The mass spectrometer detector was set at 260°C and it was operated in an electron ionization mode (EI) at 70 eV. Identification of phytosterol constituents was carried out based on the comparison of MS library data with the National Institute of Science and Technology (NIST), Gaithersburg, USA Library. The quantification of phytosterol constituents was obtained from a calibration curve of each phytosterol standard developed from five different concentrations from 30 - 1000 ppm.

3. Results and discussion

Figure 1 shows the GCMS chromatogram for major phytosterol constituents known as campesterol, stigmasterol, β-sitosterol and stigmastanol analyzed using a GCMS. The phytosterol constituents were detected in a range of 12.56 to 14.0 mins. The different time of detection for the phytosterol constituents was due to their molecular weight as shown in Table 1. These can also be observed in many studies which show that molecular weight can affect the detection time of phytosterol constituents (Yucel *et al*., 2017; Deme *et al*., 2021).

The mass spectrum for all phytosterol constituents are shown in Figures 2, 3, 4 and 5 which represent peak *a*, *b*, *c* and *d* respectively. The highest value of mass for

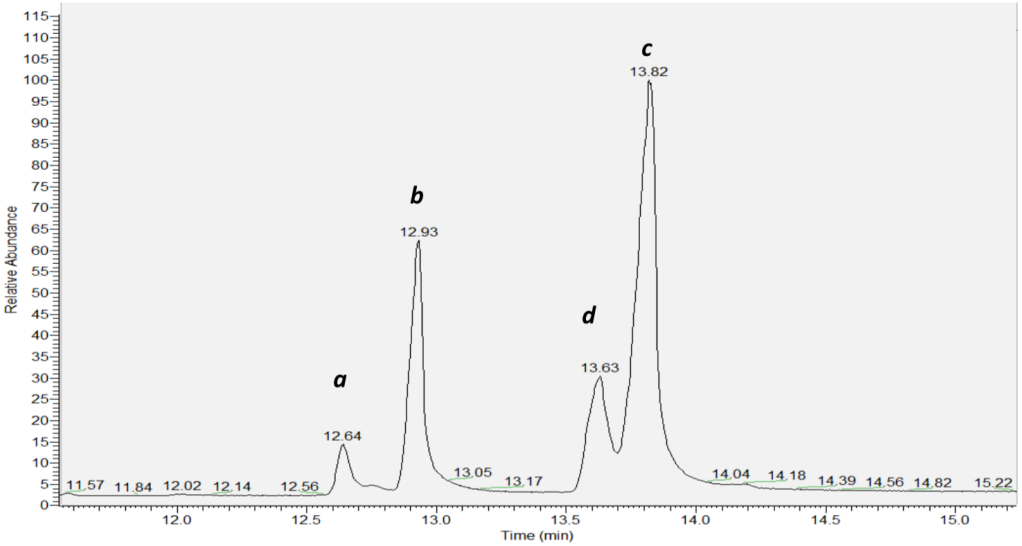

Figure 1. Major phytosterol constituents from vegetable (spinach) detected using a GCMS analyzer (*a* : campesterol; *b*: stigmasterol; *c*: β-sitosterol, *d*: stigmastanol)

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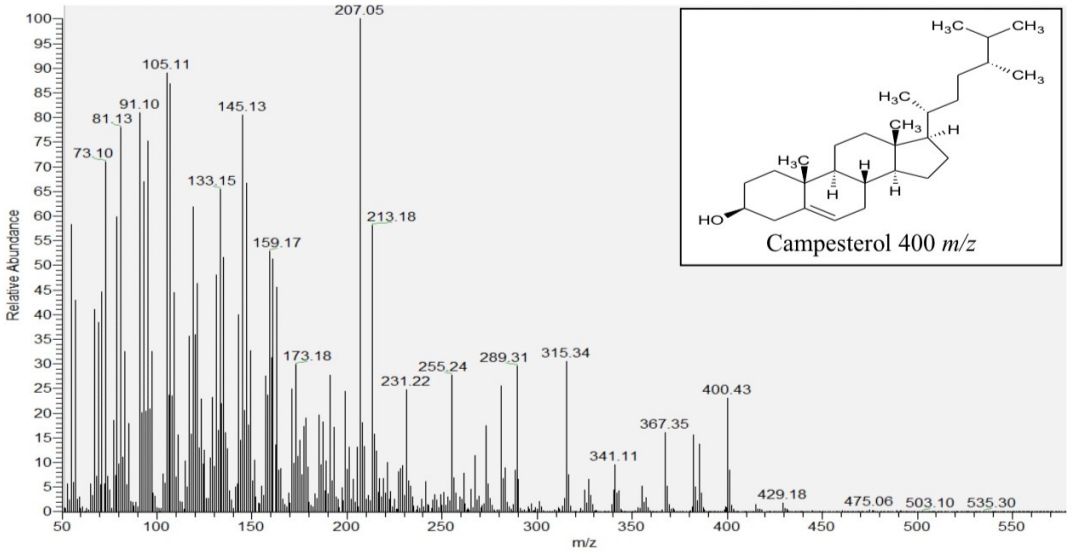

Figure 2. A mass spectrum of campesterol (peak *a*) with a mass value of 400 m/z

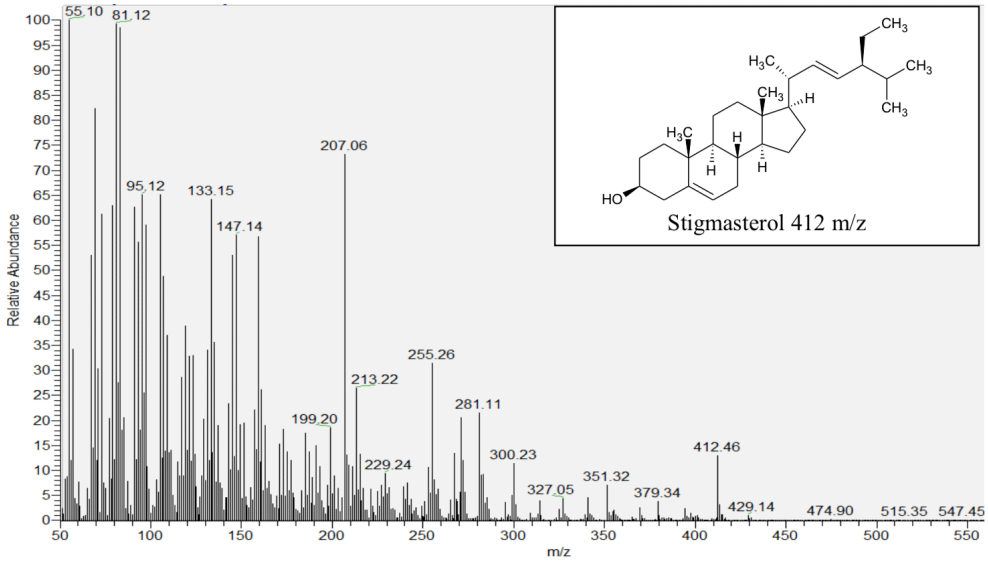

Figure 3. A mass spectrum of stigmasterol (peak *b*) with a mass value of 412 *m/z*

Table 1. Phytosterol constituents with analytes retention time and mass value detection

Retention time (R_t)	Phytosterol constituents	Mass (*m/z*)
12.64	Campesterol	400
12.93	Stigmasterol	412
13.63	β-sitosterol	414
13.82	Stigmastanol	416

the peak *a*, *b*, *c* and *d* was at 400, 412, 414 and 416 *m/z* respectively. Based on these mass values, the peaks of *a*, *b*, *c* and *d* were identified as campesterol, stigmasterol, β -sitosterol dan stigmastanol. An analyte of peak *a* was identified as campesterol due to its molecular weight of 400 *m/z* (Figure 2). Other fragments presence as m/z 213, 255, 289, 315 and 367 were usually identified as campesterol (Nakakuni *et al*., 2019) which was supported by its compound identity.

The highest mass value for peak *b* in Figure 3 mass spectrum was recorded at 412 *m/z*, which represent stigmasterol. The fragment mass value of 379 *m/z* usually represents the losses of methyl (-CH_3) and water [-H_2O] from the main skeleton of stigmasterol (Suttiarporn *et al.*, 2015). Other mass values such as 351, 327, 300, 255 *m/z* were also reported for the stigmasterol.

A mass value of 414 *m/z* represents a β-sitosterol (Figure 4). Other mass values of 396 *m/z* represent the losses of water molecule [M-H_2O] from its molecular ion of β-sitosterol structure as reported in phytosterol constituents in *Elaeagnus angustifolia* study (Azeez *et al*., 2018). Other fragments of 341, 329, 303, 273 *m/z* were observed and similar to the previous reports (Parihar and Balekar, 2017; Aliyu *et al.*, 2020). The mass values of 401, 383, 248, 233 and 215 *m/z* observed in Figure 5 were commonly reported as stigmastanol characters (Corbin *et al*., 2001; Chuanphongpanich *et al*., 2006) with the highest peak of 215 *m/z* and followed by 233 *m/z*. The highest mass value of 416 *m/z* found to support the compound that identified as stigmastanol.

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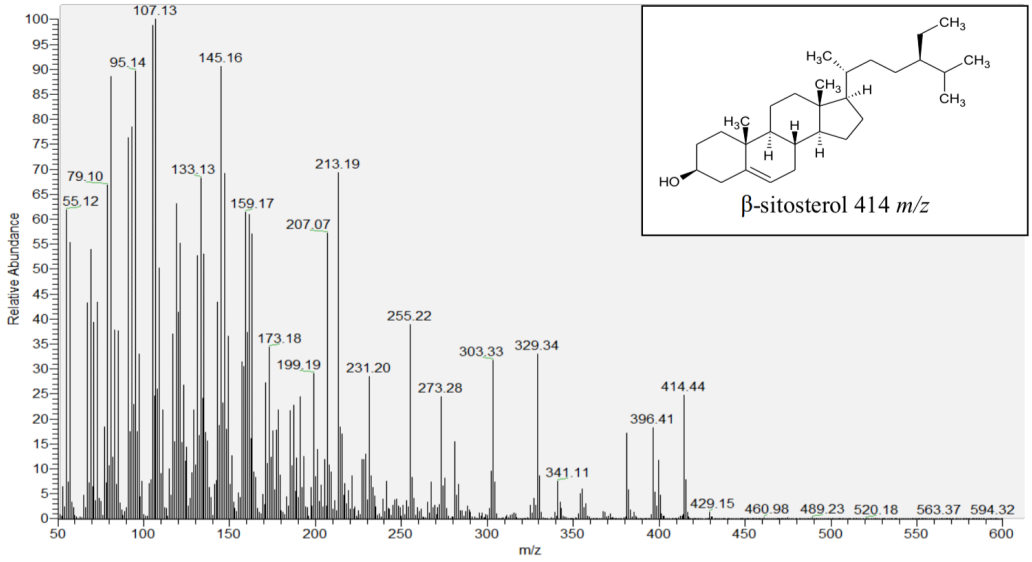

Figure 4. A mass spectrum of β-sitosterol (peak *c*) with a mass value of 414 *m/z*

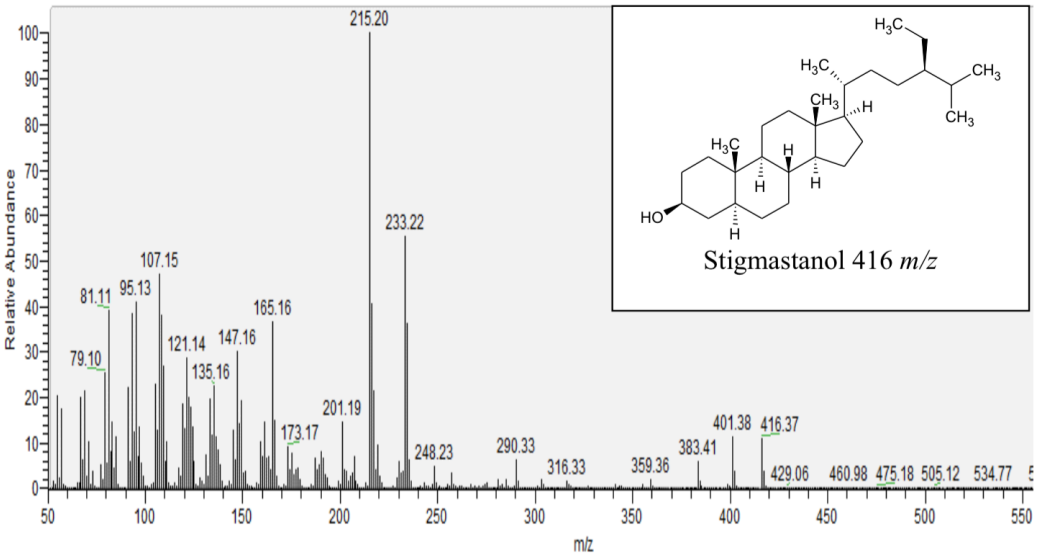

Figure 5. A mass spectrum of stigmastanol with a mass value of 416 *m/z*

4. Conclusion

Fresh vegetables have a short shelf life under certain circumstances, a proper production line for fresh vegetable-based processing is needed to overcome problems in the vegetable industry. Phytosterol constituents have been identified and these constituents are widely present in varieties of vegetables. In this study, the analysis method for phytosterol constituents was developed for campesterol, stigmasterol, β-sitosterol and stigmastanol and a GCMS analysis procedure for the selected phytosterols was established. All the phytosterol constituents were successfully detected by the GCMS analysis procedures within 12.56 to 14.00 minute retention time. This analysis is crucial and only specialized in order to determine the phytochemical contents for selective vegetables with high phytosterol constituents. This specialised protocol method can be used to monitor each batch of vegetable-based productions in vegetable factories and industries. This procedure of analysis can vitalize health and wellness food industries especially based on phytosterol constituents from vegetables.

Conflict of interest

The authors declare no conflict of interest.

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References

Aliyu, S., Basar, N., Rufa, Y. and Khamis, S. (2020). Phytochemical analysis and isolation of secondary metabolites from *Persea declinata*. *Malaysian Journal of Analytical Sciences*, 24(6), 970 – 977.

Araújo, L.B.D.C., Silva, S.L., Galvao, M.A.M., Ferreira, M.R.A., Araújo, E.L., Randau, K.P. and Soares, L.A.L. (2013). Total phytosterol content in drug materials and extracts from roots of

Acanthospermum hispidum by UV-VIS spectrophotometry. *Revista Brasileira Farmacognosia,* 23(5), 736-742. https://doi.org/10.1590/S0102-695X2013000500004

Azeez, R.A., Abaas, I.S. and Kadhim, E.J. (2018). Isolation and characterization of β-sitosterol from *Elaeagnus angustifolia* cultivated in Iraq. *Asian Journal of Pharmaceutical and Clinical Research*, 11(11), 442-446. https://doi.org/10.22159/ajpcr.2018.v11i11.29030

Bacchetti, T., Masciangelo, S., Bicchiega, V. and Ferretti, G. (2011). Phytosterols, phytostanols and their esters: from natural to functional foods. *Mediterranean Journal of Nutrition and Metabolism,* 4(3), 165–172. https://doi.org/10.3233/s12349-010-0049-0

Chuanphongpanich, S., Tuwanon, N., Buddhasukh, D., Pirakitikulr, P. and Phanichphant, S. (2006). Stanol synthesis from palm oil distillate. *Chiang Mai Journal of Science*, 33(1), 109-116.

Corbin, D.R., Grebenok, R.J., Ohnmeiss, Greenplate, J.T. and Purcell, J.P.T.E. (2001). Expression and chloroplast targeting of cholesterol oxidase in transgenic tobacco plants. *Plant Physiology*, 126(3), 1116-1128. https://doi.org/10.1104/pp.126.3.1116

Deme, T., Haki, G.D., Retta, N., Woldegiorgis, A., Geleta, M., Mateos, H. and Lewandowski, P.A. (2021). Sterols as a biomarker in tracing niger and sesame seeds oils adulterated with palm oil. *Heliyon*, 7, e06797. https://doi.org/10.1016/j.heliyon.2021.e06797

Department of Agriculture Malaysia (2020). Booklet Plantation Statistic (Sub-sector Food Plantation), p. 1 -122. Malaysia: Department of Agriculture.

Munoz, O. and Ramos, F. (2016). Quantitative analysis of phytosterols in *Aristotelia chilensis* (Maqui) leaves using GC/MS. *International Food Research Journal,* 23(2), 822-826.

Nakakuni, M., Yamasaki, Y. and Yoshitake, N. (2019). Methyl ether-derivatized sterols and coprostanol produced via thermochemolysis using tetramethylammonium hydroxide (TMAH). *Molecules*, 24 4040. https://doi.org/10.3390/molecules24224040

Parihar, G. and Balekar, N. (2017). Isolation and characterisation of stigmast-5-en-3-ol (β-sitosterol) from *Calotropis procera* latex ethyl acetate fraction for immunomodulatory activity. *International Journal of Pharmaceutical Sciences and Research*, 8 (3), 1375-1380.

Trautwein, E.A., Vermeer, M.A., Hiemstra, H. and Ras, R.T. (2018). LDL-cholesterol lowering of plant sterols and stanols—which factors influence their efficacy? *Nutrients,* 10(1262), 1-15. https://doi.org/10.3390/nu10091262

Suttiarporn, P., Chumpolsri, W., Mahatheeranont, S., Luangkamin, S., Teepsawang, S. and Leardkamolkarn, V. (2015). Structures of Phytosterols and Triterpenoids with Potential Anti-Cancer Activity in Bran of Black Non-Glutinous Rice. *Nutrients*, 7(3), 1672-1687. https://doi.org/10.3390/nu7031672

Yang, R., Xue, L., Zhang, L., Wang, X., Qi, X., Jiang, J., Yu, L., Wang, X., Zhang, W., Zhang, Q. and Li, P. (2019). Phytosterol contents of edible oils and their contributions to estimated phytosterol intake in the Chinese diet. *Foods,* 8, 334. https://doi.org/10.3390/foods8080334

Yucel, C.O., Ertas, H. and Ertas, F.N. (2017). Gas chromatographic determination and method validation of stigmasterol, β-sitosterol, campesterol and brassicasterol contents of Turkish cottonseed oil samples. *Turkish Journal of Agriculture - Food Science and Technology*, 5(11), 1274-1278. https://doi.org/10.24925/turjaf.v5i11.1274-1278.1267

https://doi.org/10.26656/fr.2017.6(S).007